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Neuronal and extraneuronal lipofuscinosis in Merino sheep grazing *Asphodelus aestivus* seeds in western Turkey

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Abstract: Neuronal and extraneuronal lipofuscinosis was determined in Merino sheep (6 sheep and 3 heads of sheep) of 2-3 years old that had grazed on *Asphodelus aestivus* seeds. Clinically, the sheep exhibited tremors, paresis, oral and nasal discharge, and severe respiratory distress. Haemorrhagic necrotic tracheitis, aspiration pneumonia, and subacute enteritis were observed in 6 sheep during macroscopic examinations. The brains of the animals were of firm consistency and yellowish brown in colour. Histological examination of the central nervous system revealed that most of the cytoplasm of neurons contained yellowish brown pigment granules. Pigmentations were particularly detected in the pons, medulla oblongata, and medulla spinalis. Similar pigment granules were also found in the liver, kidneys, intestines, adrenal glands, and heart. The histochemical and electron microscopic examinations identified the pigment as lipofuscin.

Key words: *Asphodelus aestivus*, electron microscopy, lipofuscinosis, pathology, sheep

Introduction

Lipofuscin is described as an autofluorescent lipopigment, associated with age and/or caused by the peroxidation of unsaturated lipids, and it accumulates in metabolically active cells such as neurons, all types of muscle, and cells of many organs of the body (1-3). Deposits fluoresce brown in UV light and are stainable with fat-soluble dyes, acid-fast stains, and the periodic acid-Schiff (PAS) reaction (1,3,4). Ultrastructurally, lipofuscin appears as dense amorphous autophagolysosomes packed with granules, vacuoles, and lipid globules (1).

Many pasture plants have been reported to have an influence on the development of acquired lipofuscinosis (5-7). Neuronal and extraneuronal lipofuscinosis have been reported in sheep on pastures in the south of Western Australia with predominantly *Trachyandra divaricata* (6) and in South Africa with *T. divaricata* (7) and *Trachyandra laxa* (5). The pigmentation was also produced experimentally in sheep and horses by feeding them *T. laxa* (5). Moreover, in a recent study, similar pigmentation associated with consumption of *Asphodelus aestivus* leaves in western Turkey was also described (8). The common clinical signs reported in these publications (5-8) were characterised by severe neurological signs such as severe tremors, convulsion, and paralysis. Besides these signs, massive accumulation

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of lipofuscin in the neurons of the central nervous system (CNS), in the autonomous ganglions, and in some extraneuronal tissues was observed histopathologically.

The object of this study was to describe histopathological and electronmicroscopic findings in neuronal and extraneuronal lipofuscinosis presumably due to *A. aestivus* seeds in sheep during the dry season in western Turkey.

Description and distribution of *Asphodelus aestivus*

Description: A. aestivus is a perennial plant up to 2 m long with leaves measuring 25-40 cm × 15-30 mm (Figure 1A and 1B). It is inflorescence branched with dense-flowered racemes and bracts, which are 5-15 mm and scarious or greenish in colour. The pedicels are jointed at the middle, perianth are segments of white with a pink or brownish midvein, 10-15 mm stamens more or less equal; capsules are 5-7 mm, obovoid, and transversely wrinkled (9).

Distribution: Mediterranean area, Portugal, Corsica, Crete, Balearics, France, Greece, Spain, Italy, Former Yugoslavia, Sicily, Islands of Aegean, and Turkey (10).

Materials and methods

The 6 Merino sheep (4 alive, cases 1-4; 2 dead, cases 5 and 6) between 2 and 3 years old and 3 heads of sheep (cases 7-9) were delivered to the Department of Pathology at the Veterinary Faculty of Adnan Menderes University. Using sodium pentobarbital, 4 sheep were euthanised, and then necropsies were performed on 6 sheep and 3 heads of affected sheep. For histopathologic examinations, tissue specimens, taken from the CNS and other tissues (liver, kidneys, adrenal glands, forestomach, intestines, muscle, heart, lung, spleen, lymph nodes, and eyes) were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin wax. Sections were cut to a thickness of 5-6 µm and stained with haematoxylin and eosin (H&E). The selected sections were stained by the PAS reaction, Schmorl's method, a long Ziehl-Neelsen stain for lipofuscin, and melanin removal method II for melanin (4,11). In addition, the unstained deparaffinised sections of the brain, spinal cord, and other tissues were examined using fluorescence microscopy.

Small pieces of the brain stem were fixed in 2% glutaraldehyde in phosphate buffered saline for 48 h

Figure 1. A) *Asphodelus aestivus*, B) *Asphodelus aestivus* seeds.

at 4 °C. They were then postfixed in osmium tetroxide and embedded in Epon. The ultrathin sections (7-10 Å) were stained with lead citrate and uranyl acetate for transmission electron microscopy (4).

Results

Clinical signs

The outbreak occurred during the dry season, in a flock of 220 Merino sheep that grazed on a pasture in Aydın Province, of which 38 died in June 2006. In the pasture the vegetation was sparse and dominated by *A. aestivus* seeds. The owner reported that the sheep were exposed to seeds of *A. aestivus*, and the animals had to consume the seeds, which were the main source of feed available. The owner also mentioned that the *A. aestivus* seeds were not consumed during previous years. Clinical examination of the 4 sheep showed tremors, paresis, diarrhoea, oral and nasal discharge, and respiratory distress. The body temperature of the sheep varied between 39.5 and 41 °C.

Macroscopic findings

Macroscopic examination in sheep revealed that the meninges were opaque, thick, and hyperaemic. The cerebrum had firm consistency and showed a yellowish brown discolouration (Figure 2A). Less than 50% of the oesophagus from the sheep was filled with ruminal contents. The forestomach had content with normal consistency, and it contained high amounts of *A. aestivus* seeds (Figure 2B). The mesenterium and omentum were diffusely haemorrhagic. Haemorrhages were also observed on the serosa of the small and large intestines. The liver and kidneys were swollen and showed a firm consistency, and also a reddish brown discolouration in cut surfaces (Figure 2C). Laryngeal mucosa was haemorrhagic and covered by pseudomembranes. The trachea was filled with foamy exudates and aspirated ruminal contents. Its mucosa was also haemorrhagic. Furthermore, aspiration pneumonia was observed in 6 sheep.

Microscopic findings

Light microscopy: Histologic examination of the CNS indicated that most of the cytoplasm of neurons contained yellowish brown pigment granules (Figure 3A). The pigment granules were particularly abundant in the motor neurons of the pons, medulla oblongata, midbrain (crus cerebri, tegmentum, and tectum mesencephali), and medulla spinalis, and were found to a lesser extent in the cerebrum. The amount of pigment in the neurons varied considerably. In the cytoplasm of some neurons, pigment granules were diffusely scattered and located eccentrically in their

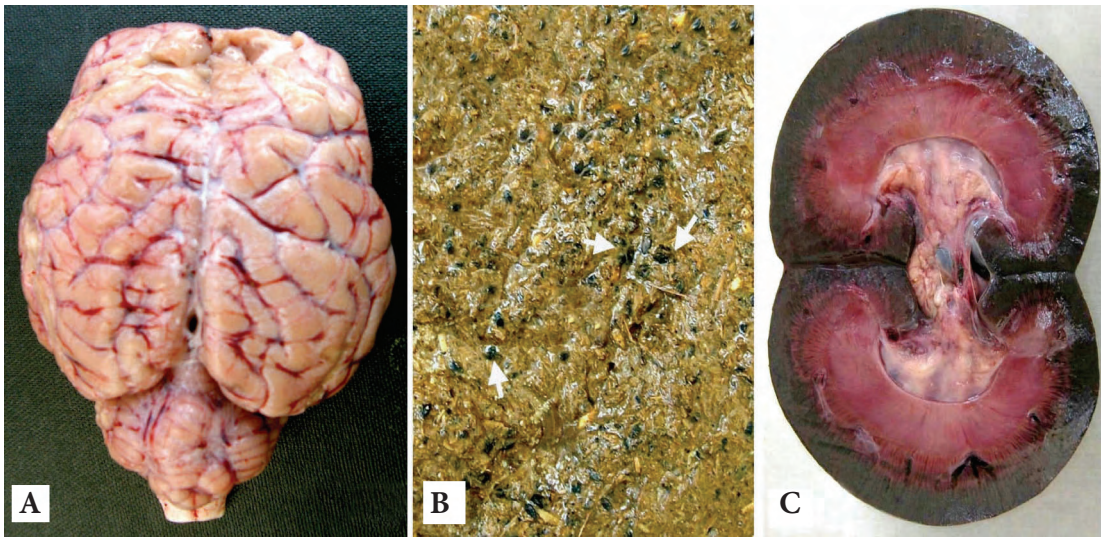

Figure 2. A) The cerebrum exhibited a slight yellowish brown discolouration with blurred meninx (case 5). B) Forestomach (rumen) contained an excess amount of *Asphodelus aestivus* seeds (arrows) (case 4). C) The kidney showed reddish brown discolouration in the cutting surface (case 2).

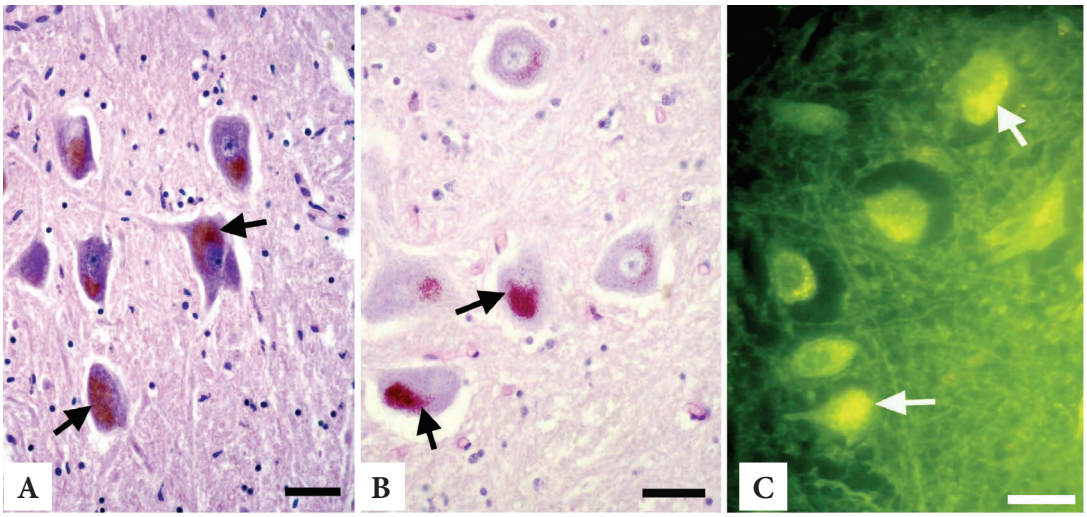

Figure 3. A) The yellowish brown pigment granules were abundantly present in neurons of the medulla oblongata (arrows, case 5). H&E. Bar = 40 µm. B) The pigment granules were stained positively with PAS, pons (arrows, case 5). Bar = 40 µm. C) Autofluorescence of lipofuscin in the cytoplasm of neurons (arrows), medulla oblongata (case 5). Bar = 50 µm.

perikarya. In others neurons, there were only small aggregates of pigment. Distribution of the pigment granules in the CNS and visceral organs of the sheep is presented in the Table. The granules were positively stained with PAS (Figure 3B), Schmorl's method, and the long Ziehl-Neelsen stain for lipofuscin. Lipofuscin also exhibited strong autofluorescent on fluorescence microscopy (Figure 3C). The pigment granules were neither stained with the Turnbull blue method for hemosiderin, nor with melanin removal method II for melanin. Several neurons exhibited degenerative changes by oedematous swelling of the cytoplasm as well as by pericellular oedema. Microgliosis and satellitosis were found accompanying those neurons. Perivascular haemorrhages were also found as a remarkable finding. The pigment granules were not observed in Purkinje cells of the cerebellum of all animals.

In the liver, cytoplasmic pigment granules with similar staining properties were present in both hepatocytes and Kupffer cells. Mild to moderate fatty changes and periacinar necrosis were also observed in the liver. The accumulated pigment granules and necrosis were very obvious in the proximal tubular epithelial cells of the kidney (Figure 4A) together with hyperaemia and interstitial haemorrhages in both the cortex and medulla. The pigment granules were also identified in the adrenal cortex. Similar yellow-brown pigment granules were found in the myocytes of the heart (Figure 4B). The autonomic plexuses of the submucous ganglions at the larynx were remarkably full of similar pigment granules. Aspiration pneumonia and ruminal content mixed with bacterial clusters were observed in the lumen of the bronchi and bronchioles of the lungs. In the intestines, similar lipofuscin granules were occasionally seen in the myenteric plexus of the jejunum and ileum (Figure 4C). Scattered macrophages with cytoplasmic pigment granules were also found in the lamina propria of the jejunum and ileum (Figure 4D). Desquamation and necrosis of the villus epithelium, and a small amount of eosinophil leucocyte infiltration with lymphocyte infiltration of the lamina propria, were also found in the intestines.

Electron microscopy

The perikaryal cytoplasm of neurons contained clusters of irregularly shaped, electron-dense bodies measuring 0.5-2.5 µm along their greatest dimension. Variably sized, electron-lucent vacuoles were situated within or at the periphery of some of these bodies. Some of them clearly possessed granular and membranous substructures, and a limiting membrane was evident around many of them (Figure 5A and 5B).

Table. Distribution of lipofuscin pigment granules in various tissues of sheep.

	Central nervous system						Visceral organs				
Animal no.	Cerebrum	Cerebellum	Midbrain	Pons	Medulla oblongata	Medulla spinalis	Liver	Kidneys	Adrenal glands	Heart	Intestines
1	+	-	+	++	++	+	+	++	-	++	++
2	++	-	++	+++	+++	++	+++	+++	++	+++	+++
3	++	-	++	+++	++	+	++	++	+	+++	+++
4	+	-	++	+++	+++	+	+++	+++	-	+++	++
5	+	-	+++	+++	+++	++	+++	+++	++	++	+++
6	+	-	++	+++	++	+	++	++	-	+	++
7	++	-	++	+++	+++	+	n	n	n	n	n
8	+	-	++	+++	+++	+	n	n	n	n	n
9	+	-	+	+++	++	-	n	n	n	n	n

(-, none; +, slight; ++, moderate; +++, severe; n, not examined.)

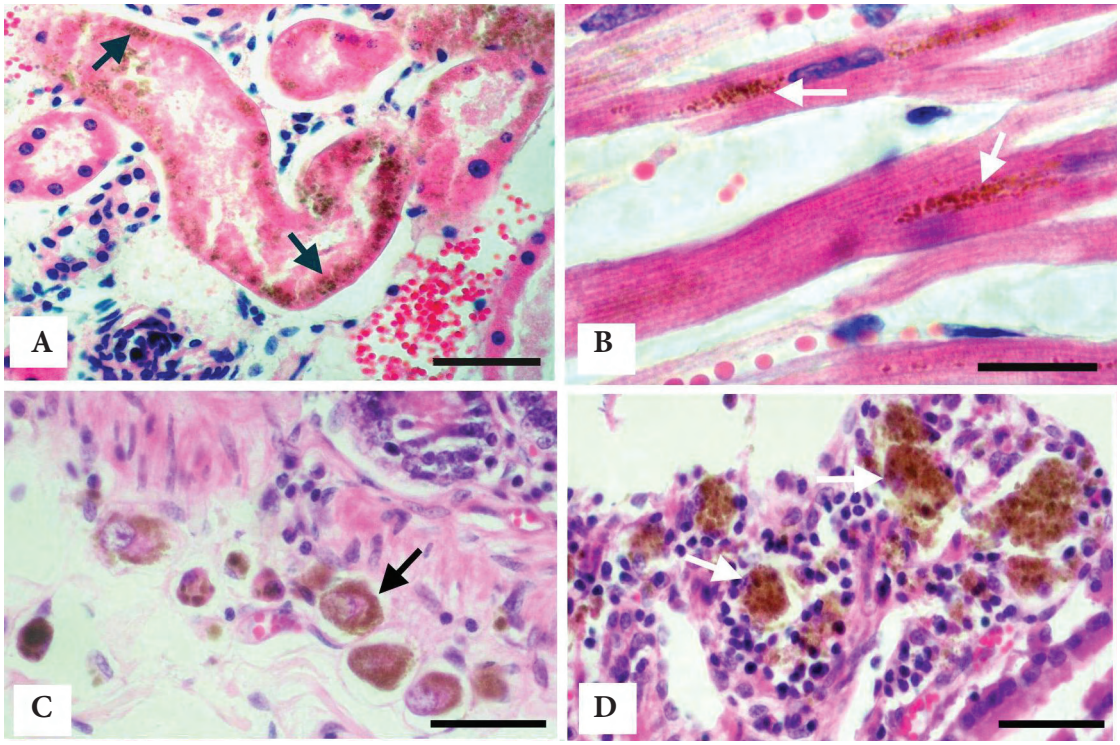

Figure 4. A) The kidney showed the yellowish brown pigment granules and necrosis in the epithelium of the proximal tubules (arrows, case 2). H&E. Bar = 40 µm. B) The yellow-brown pigment granules in the myocytes of the heart (arrows, case 3). H&E. Bar = 20 µm. C) The pigment granules in autonomic ganglionic neurons of the jejunum (arrow, case 2). H&E. Bar = 40 µm. D) The macrophages contained the yellow-brown pigment granules (arrows, case 2), jejunum. H&E. Bar = 40 µm.

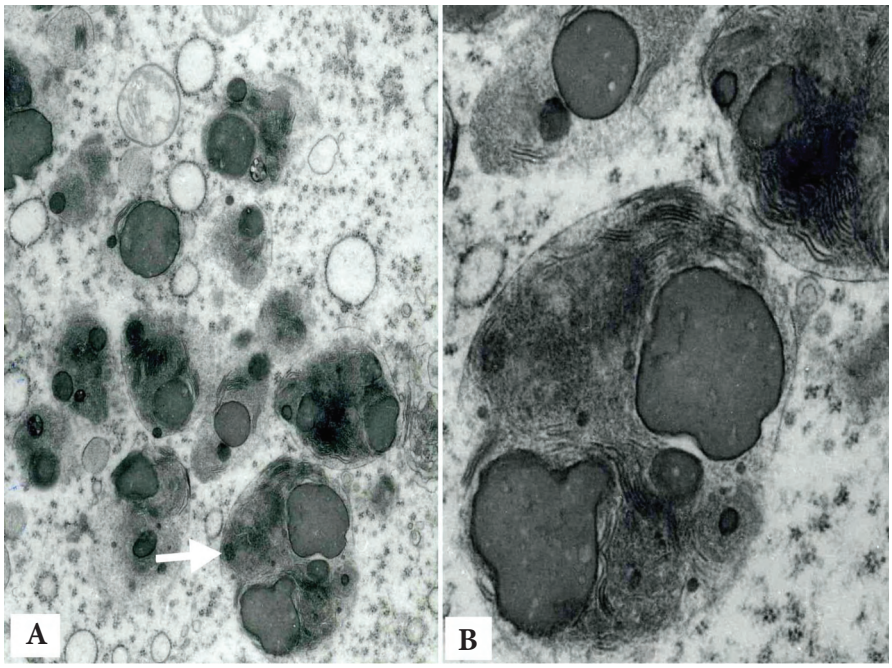

Figure 5. Transmission electron micrograph with limiting membrane electron-dense lipofuscin granules in neuron of the pons (arrow, case 5): A) 6300×, B) 16,000×.

Discussion

Neuronal and extraneuronal lipofuscinosis, as presented in this study, was a remarkable finding in sheep after ingestion of *A. aestivus* seeds. The outbreak took place during the dry season, because most of the other plants had withered. Thus, the pasture contained no fresh feed, but an excess of the *A. aestivus* seeds. A similar severe neurotoxic syndrome characterised by neurovisceral lipofuscinosis in sheep has already been described in Italy, where 4 toxic plants (*Cistus*, *Pistacia*, *Quercus*, and *A. aestivus*) were mentioned as possible aetiological agents (12). Since the first 3 plants cited did not exist in our pasture, we concluded that *A. aestivus* was responsible for the neuropathology described in the literature. In a recent study, similar pigmentation associated with the consumption of leaves of *A. aestivus* predominant in the pastures of Aydın Province in western Turkey was described (8). The neuronal lipofuscinosis had also been reported in sheep due to *T. divaricata* (6,7) and *T. laxa* (5) poisoning. In addition, *A. aestivus* is morphologically similar to those 2 plants, and taxonomically it belongs to the same family as *Trachyandra* sp. (*Asphodelaceae* = Liliaceae). To our knowledge, this is the first report on the neurotoxicity of *A. aestivus* seeds in sheep.

Lipofuscin and ceroid are usually held responsible for impaired cellular vitality, via oxidative damage and the accumulation of irreversible fluorescent products of lipid peroxidation (1,2). Discrimination between lipofuscin and ceroid lipofuscin could be done by histochemical, fluorescence, and electronmicroscopic examinations (1,2,4). In the present study, the pigment reacted with PAS, Schmorl's, and long Ziehl-Neelsen staining, and it was also strongly autofluorescent. Based on the results, the pigment was distinguished from ceroid lipofuscin by the negative reaction using Schmorl's method. Ultrastructurally, lipofuscin pigments with a granular and a lamellar structure surrounded by a membrane were distinguished. These results were consistent with previous studies (2,3,7).

Previous phytochemical investigations performed on the leaves of *Asphodelus* species have resulted in the isolation of anthranoids (anthraquinones and bianthrone derivatives), flavonoids, and triterpenes (13-18). Although the relationship between neurovisceral lipofuscinosis and the anthranoids or other metabolites of *A. aestivus* could not be evaluated in the present study, these observations prompted us to investigate the secondary metabolites

of *A. aestivus*, which may have led to poisoning in farm animals.

In the sheep, aspiration pneumonia was found as the final reason of death. As this is a rather rare finding in general, we consider this as a pathognomonic sign in our sheep due to laryngeal paralysis (19). This situation most probably occurred due to the lipofuscinosis and degeneration of autonomic plexuses within the larynx submucosa.

The purpose of this study was to describe the histopathologic and electronmicroscopic findings in neurovisceral lipofuscinosis by seeds of *A. aestivus* in sheep grazed on pasture in western Turkey. The pathogenesis of neuronal and extraneuronal lipofuscinosis was presumably due to the *A. aestivus* seeds. We recommend that this outcome should be confirmed by experimental feeding in future studies.

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